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α-SNAP but not γ-SNAP is required for ER-Golgi transport after vesicle budding and the Rab1-requiring step but before the EGTA-sensitive step

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SUMMARY

***N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs) have been implicated in diverse vesicular transport events; yet their exact role and site of action remain to be established. Using an established in vitro system, we show that antibodies against α-SNAP inhibit vesicle transport from the ER to the *cis*-Golgi and that recombinant α-SNAP enhances/stimulates the process. Cytosol immunodepleted of α-SNAP does not support normal transport unless supplemented with recombinant α-SNAP but not γ-SNAP. In marked contrast, cytosol immunodepleted of γ-SNAP supports ER-Golgi transport to the normal level. Neither antibodies against γ-SNAP nor recombinant γ-SNAP have any effect on ER-Golgi transport. These results clearly establish an essential role for α-SNAP but not γ-SNAP in ER-Golgi transport. When the transport assay is performed with cytosol immunodepleted of α-SNAP, followed by incubation with cytosol immunodepleted of a COPII subunit, normal transport is achieved. In marked contrast, no transport is detected when the assay is first performed with cytosol depleted of the COPII subunit followed by α-SNAP-depleted cytosol, suggesting that α-SNAP is required after a step that requires COPII (the budding step). In combination with cytosol immunodepleted of Rab1, it is seen that α-SNAP is required after a Rab1-requiring step. It has been shown previously that EGTA blocks ER-Golgi transport at a step after vesicle docking but before fusion and we show here that α-SNAP acts before the step that is blocked by EGTA. Our results suggest that α-SNAP may be involved in the pre-docking or docking but not the fusion process.**

Key words: SNAP, Vesicular transport, Docking/fusion, ER, Golgi

INTRODUCTION

Protein transport between membrane compartments along the secretory pathway is primarily mediated by vesicles that bud from a donor compartment followed by targeting to, docking onto and fusion with an acceptor membrane (Pryer et al., 1992; Rothman, 1994; Rothman and Wieland, 1996; Schekman and Orci, 1996). NSF and SNAPs have been implicated in diverse transport events in the vesicle docking/fusion stage and it has been proposed that they function as general fusion proteins (Whiteheart and Kubalek, 1995). Sec18p (Eakle et al., 1988) is the yeast counterpart of NSF (Wilson et al., 1989). Three mammalian forms of SNAPs have been identified and are referred to as α-, β-, and γ-SNAP, respectively (Clary et al., 1990; Whiteheart et al., 1993). β-SNAP is brain specific and highly homologous to α-SNAP, while α- and γ-SNAPs are widely distributed and exhibit 25% amino acid identity. Sec17p is the yeast SNAP and can be functionally substituted for mammalian α-SNAP (Clary et al., 1990; Griff et al., 1992). To account for the specificity of vesicle transport, the SNAP receptor (SNARE) hypothesis proposes that vesicles for a defined transport event have unique integral membrane proteins termed v-SNAREs (vesicle SNAREs) that will interact specifically with the cognate SNAREs on the target membrane (t-SNAREs) (Ferro-Novick and Jahn, 1994; Pfeffer, 1996; Rothman, 1994; Rothman and Warren, 1994; Rothman and Wieland, 1996; Scheller, 1995; Söllner et al., 1993). Synaptobrevins (or VAMPs) function as v-SNAREs associated with the synaptic vesicles while syntaxin 1 and SNAP-25 function as t-SNAREs on the presynaptic membrane (Scheller, 1995; Söllner et al., 1993; Südhof, 1995). The correct pairing between v- and t-SNAREs then triggers the recruitment of SNAPs and subsequently NSF onto the paired SNAREs, leading to the formation of the v-SNAREs-t-SNAREs-SNAPs-NSF fusion complex (or 20S SNARE complex). This complex is intimately associated with vesicle fusion to the target membrane through a process that involves ATP hydrolysis by NSF and the disassembly of the 20S complex. The pairing between the v- and t-SNAREs and the formation of the SNARE complex can be regulated by other proteins such as members of the Rab/Sec4p/Ypt1p family of small GTPases (Lian et al., 1994; Novick and Brennwald, 1993; Nuoffer and Balch, 1994; Simons and Zerial, 1993; Sögaard et al., 1994), and/or other types of proteins that interact with either v-SNAREs (such as synaptophysin) (Calakos and Scheller, 1994; Edelmann et al., 1995) or t-SNAREs (such as Sec1p-like proteins) (Scheller,

1995; Südhof, 1995) and prevent their pairing with the cognate SNAREs, providing additional control over the specificity of vesicle docking/fusion.

The functional importance of SNAPs and NSF and the precise stage at which they act remain to be experimentally examined for many transport events (Rothman, 1994; Rothman and Wieland, 1996; Whiteheart and Kubalek, 1995). Two recent studies have suggested alternative modes of action for α-SNAP that are at variance with the SNARE hypothesis. In one study, it was revealed that NSF is associated with highly purified clathrin-coated vesicles and that clathrin-coated vesicles contain sufficient proteins to incorporate α-SNAP into a 20S-SNARE complex (Steel et al., 1996). In a separate investigation of the potential roles of Sec17p and Sec18p in homotypic fusion of yeast vacuoles, it was seen that Sec17p and Sec18p actions precede the step employing Ypt7p and that Sec18p-driven release of Sec17p can actually precede the docking and fusion steps, suggesting that Sec17p and Sec18p may function in a pre-docking stage during vacuole fusion (Mayer et al., 1996). These observations prompted us to investigate the functional importance and the stage of action of α-SNAP and γ-SNAP in ER-Golgi transport using a well-established in vitro system. Our study demonstrates that α-SNAP but not γ-SNAP is absolutely required for ER-Golgi transport at a step after vesicle budding and the Rab1-requiring step but before the EGTA-sensitive stage.

MATERIALS AND METHODS

Recombinant α-SNAP and γ-SNAP

Recombinant hexahistidine-tagged α-SNAP was prepared as described (Whiteheart et al., 1993) with some minor changes. Briefly, the pQE-9 vector containing the α-SNAP coding region (kindly provided by Dr James E. Rothman, Memorial Sloan-Kettering Cancer Center) was transformed into competent M15 *Escherichia coli* strain harbouring the pREP4 plasmid coding for high levels of LacI repressor. Cultures of cells were induced with 1 mM iso-propyl thio-galactoside (IPTG) for 12 hours at room temperature and centrifuged at 5,000 rpm in a GSA rotor (Sorvall, Du Pont). Cells were lysed in cracking buffer (100 mM Hepes-KOH, pH 7.3, 5 mM $MgCl_2$, 500 mM KCl, 2 mM β-mercaptoethanol, 1 mM PMSF) by sonication. After centrifugation at 15,000 rpm in a SS-34 rotor for 30 minutes, the supernatant was passed over a column of Ni^{2+}-NTA resin (Qiagen) and washed with 50 column volumes of 25 mM imidazole in elution buffer (20 mM Hepes-KOH, pH 7.3, 200 mM KCl, 2 mM β-mercaptoethanol, 10% glycerol). Bound recombinant α-SNAP was eluted with 250 mM imidazole in elution buffer. For transport assay, recombinant α-SNAP was dialysed against 25 mM Hepes-KOH, pH 7.0, 125 mM KOAc. For the preparation of recombinant hexahistidine-tagged γ-SNAP, a EST clone (GenBank accession number N39674) encoding full-length human γ-SNAP was used in a PCR reaction with oligo A: 5′ GGGGGATCCGCGGC-TCAGAAGATAAACGAGGG, and oligo B: 5′ TCCCAAGCTTGC-GGCCGCAGTTTTTTTTTTT. The PCR product was blunt-ended and then digested with *Bam*HI and then cloned into the *Bam*HI and *Sma*I sites of pQE-30 vector. The ligation reaction was transformed into *E. coli* strain M15 and N-terminal hexahistidine-tagged γ-SNAP was purified as above.

Antibodies

New Zealand White rabbits were immunized with 300 μg of recombinant proteins emulsified in complete Freund's adjuvant. Booster shots of the recombinant proteins in incomplete adjuvant were administered at two week intervals. Rabbits were bled 10 days after third and subsequent boosts of antigens. Recombinant proteins conjugated to CNBr-activated Sepharose 4B were used to affinity-purify antibodies from the serum as described (Lowe et al., 1996). Antibodies against α-SNAP, γ-SNAP, and the mammalian homologue of yeast Sec13p (mSEC13) were prepared in this way. The expression, purification of mSEC13 and antibody preparation have been recently reported (Tang et al., 1997). Monoclonal antibody (m4D3c) against Rab1b was kindly provided by Dr William E. Balch, The Scripps Research Institute (Plutner et al., 1991). When rat liver cytosol was analysed by immunoblot (Fig. 1), a single protein of about 25, 30, or 36 kDa was specifically recognized by antibodies against Rab1, α-SNAP, or γ-SNAP, respectively.

In vitro ER to *cis*-Golgi transport assays

The biochemical assay to measure transport of VSV-G protein from the ER to the *cis*-Golgi was performed as described earlier (Beckers et al., 1987; Davidson and Balch, 1993). Briefly, NRK or CHO 15B cells were grown on 10 cm Petri dishes to form a confluent monolayer and infected with a temperature sensitive strain of the vesicular stomatitis virus, VSVts045. The cells were pulse labelled with [^{35}S]methionine at the restrictive temperature and mechanically permeabilized on ice. 5 μl (~1-2× 10^5 cells) of semi-intact cells were then incubated in an assay cocktail of 40 μl containing (in final concentrations) 25 mM Hepes-KOH, pH 7.2, 90 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, 0.2 i.u. of rabbit muscle creatine phosphokinase, 25 μg rat liver cytosol (Davidson and Balch, 1993). Additional reagents were added as indicated in Results. For a standard assay, samples were incubated for 90 minutes at 32°C and transport terminated by transfer to ice. The membranes were collected by a brief spin, solubilized in 0.1% SDS and digested overnight with endo H. Subsequently, 5× concentrated gel sample buffer was added and the samples were separated on 7.5% SDS-PAGE. Autoradiographs were quantified with a Phosphor Imager (Molecular Dynamics). In two stage transport assays, membranes were pelleted by a 20 second centrifugation at full speed in an Eppendorf table top centrifuge after stage I incubation and subsequently resuspended in fresh assay cocktail by repeated pipetting and reincubated at 32°C.

Immunodepletion of rat liver cytosol

Rat liver cytosol was prepared as previously described (Davidson and Balch, 1993). Immunopurified antibodies against α-SNAP, γ-SNAP, mSEC13, Rab1 (m4D3c) and haemagglutinin (HA; as mock control), respectively, were coupled to CNBr-activated Sepharose 4B (Pharmacia) as follows: 100 μl antibodies (at least 4-5 mg/ml) were dialyzed against 100 mM $NaHCO_3$, pH 8.3, 500 mM NaCl and incubated with 100 μl activated Sepharose overnight at 4°C. The remaining active groups on the beads were blocked with 100 mM Tris-HCl, pH 8, for 2 hours at ambient temperature. The beads were then washed three times with each of alternating wash buffers A (100 mM KOAc, pH 4, 500 mM NaCl) and B (100 mM Tris-HCl, pH 8, 500 mM NaCl) followed by three washes with 25 mM Hepes, pH 7.4, 125 mM KOAc. The beads were then incubated overnight with 100 μl rat liver cytosol, spun down and the supernatants tested for depletion of the respective proteins by quantitative immunoblotting. The depleted cytosols were aliquotted, flash-frozen in liquid nitrogen and stored at –80°C.

RESULTS

Polyclonal antibodies against α-SNAP inhibit while recombinant α-SNAP enhances/stimulates ER-Golgi transport

Although α-SNAP and γ-SNAP have been proposed to function as general transport factors and the yeast Sec17p has

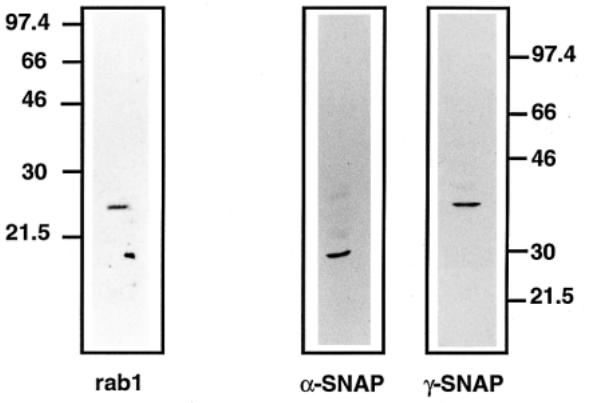


Fig. 1. Characterization of antibodies against Rab1, α-SNAP and γ-SNAP. 7.5 μg of rat liver cytosol was resolved by SDS-PAGE and transferred to a filter. The blot was probed with the respective antibodies to detect Rab1, α-SNAP and γ-SNAP.

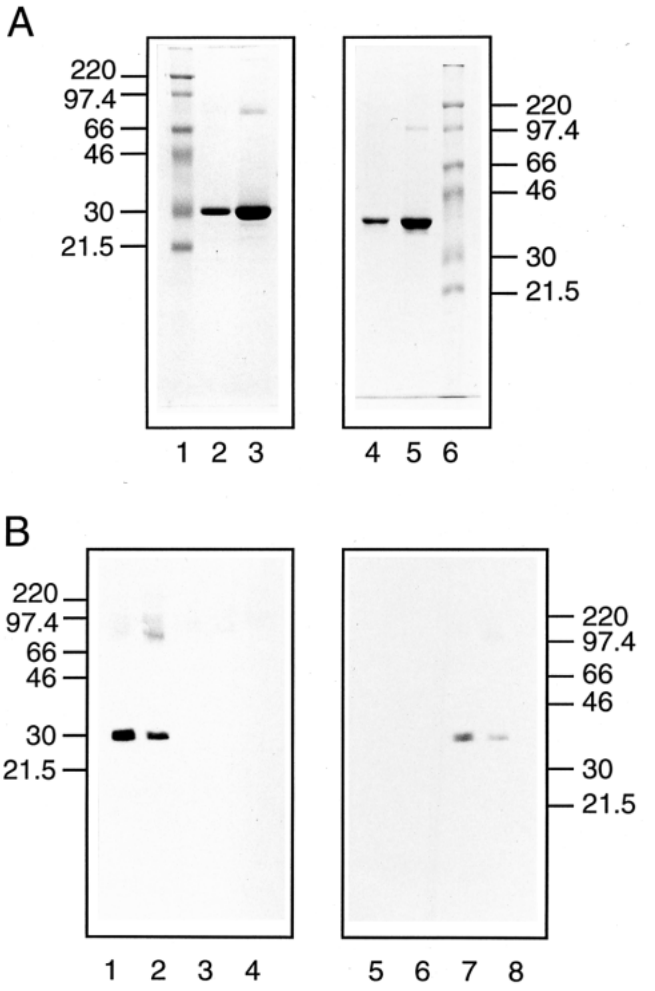


Fig. 2. Preparation of recombinant α-SNAP and γ-SNAP (A) and antibodies that are specific for α-SNAP or γ-SNAP (B). (A) 0.5 μg (lane 2), 2 μg (lane 3) α-SNAP, 0.5 μg (lane 4), and 2 μg (lane 5) γ-SNAP were resolved by SDS-PAGE and stained with Coomassie Blue. (B) 100 ng (lanes 1, 5), 20 ng (lanes 2, 6) α-SNAP, and 100 ng (lanes 3, 7), 20 ng (lanes 4, 8) γ-SNAP were analysed by immunoblot using antibodies against α-SNAP (lanes 1-4) or γ-SNAP (lanes 5-8).

been demonstrated to be essential for ER-Golgi and other transport events (Graham and Emr, 1991; Pryer et al., 1992; Whiteheart and Kubalek, 1995), direct demonstration of the functional importance of α-SNAP or γ-SNAP in ER-Golgi transport in mammalian cells has been lacking. To establish their functional importance, we have expressed and purified recombinant α-SNAP and γ-SNAP (Fig. 2A). Antibodies against α-SNAP and γ-SNAP were prepared and affinity-purified (Fig. 2B). We first examined whether affinity-purified rabbit polyclonal antibodies against α-SNAP have any inhibitory effect on ER-Golgi transport of vesicular stomatitis virus envelope G protein using the well-established in vitro transport system with perforated NRK and CHO 15B mutant cells (Balch et al., 1994; Beckers et al., 1987; Davidson and Balch, 1993; Plutner et al., 1992; Schwaninger et al., 1991). The in vitro transport with NRK cells measures the conversion of endoglycosidase H (endo H) sensitive ER form to the endo H resistant *cis*/medial Golgi form of G protein, while conversion of endo D resistant ER form to endo D sensitive *cis*-Golgi form of G protein was measured in perforated CHO 15B cells. As shown in Fig. 3c, inclusion of 2.5 μg of antibodies against α-SNAP inhibited the normal transport (b) almost to background levels (a) in both cell types. Furthermore, normal transport could be enhanced almost to the maximal levels by the inclusion of 1 μg of recombinant α-SNAP (d). Control antibodies, such as the monoclonal antibody against the HA epitope (f) had no effect on the transport. Consistent with our recent results (Subramaniam et al., 1996), the recombinant cytoplasmic domain of GS28, a *cis*-Golgi SNARE, inhibited transport to background levels (e). Fig. 4 details the dose curve of the inhibitory effect of α-SNAP antibodies (A) and the enhancing/stimulating activity of recombinant α-SNAP (B). Inclusion of 1 μg of α-SNAP antibodies inhibited transport to 50% of the standard transport levels while inclusion of 2 μg or more of α-SNAP antibodies totally inhibited transport. A normal transport reaction usually resulted in 65-75% of the pulse-labeled G protein being transported. Supplementing an increasing amount of recombinant α-SNAP gradually enhanced/stimulated the efficiency of transport with a maximal effect when 0.2 μg or more of recombinant α-SNAP was added. Excess amounts of recombinant α-SNAP (1-5 μg) had no adverse effect on ER-Golgi transport. These results clearly established that antibodies against α-SNAP can quantitatively inhibit ER-Golgi transport while recombinant α-SNAP has an enhancing/stimulating effect on ER-Golgi transport, suggesting that α-SNAP is an important but limiting factor in the in vitro transport system.

α-SNAP is necessary for ER-Golgi transport

As an alternative and independent approach to establish the functional importance of α-SNAP, we have established that α-SNAP is necessary for ER-Golgi transport using cytosol that had been immunodepleted of α-SNAP (Fig. 5). Fig. 5A shows the amounts of α-SNAP in the cytosols that have been mock-depleted (lane 1) or immunodepleted with antibodies against α-SNAP (lane 2), mSEC13 (lane 3), Rab1 (lane 4), or γ-SNAP (lane 5) and it is obvious that α-SNAP is specifically depleted with antibodies against α-SNAP. Fig. 5B shows the dose-curve

of increasing amounts of mock- and α-SNAP-depleted cytosol on the in vitro transport reaction. As compared to the mock-depleted cytosol (filled squares), α-SNAP depleted cytosol (open squares) exhibited a dramatic defect in supporting the transport. For example, 30 μg of mock-depleted cytosol can support transport to the normal level while an assay with the same amount of α-SNAP-depleted cytosol resulted in only background levels of transport (arrow). To firmly establish that the defective transport activity of α-SNAP-depleted cytosol was due to the absence of α-SNAP, transport was performed with 30 μg of the α-SNAP depleted cytosol supplemented with increasing amounts of recombinant α-SNAP (Fig. 5C, open squares). The transport activity of the α-SNAP depleted cytosol could be restored to the standard levels by the addition of about 0.2-0.25 μg of recombinant α-SNAP. Similar to control cytosol supplemented with 0.2 μg or more α-SNAP (Fig. 3), additional amounts of α-SNAP (above 0.3-0.4 μg) similarly enhanced transport to the maximal levels with the α-SNAP depleted cytosol. Under identical conditions, α-SNAP depleted cytosol could not be rescued by recombinant γ-SNAP to support normal transport (Fig. 5C, filled squares). These results firmly establish an absolute requirement for α-SNAP in ER-Golgi transport.

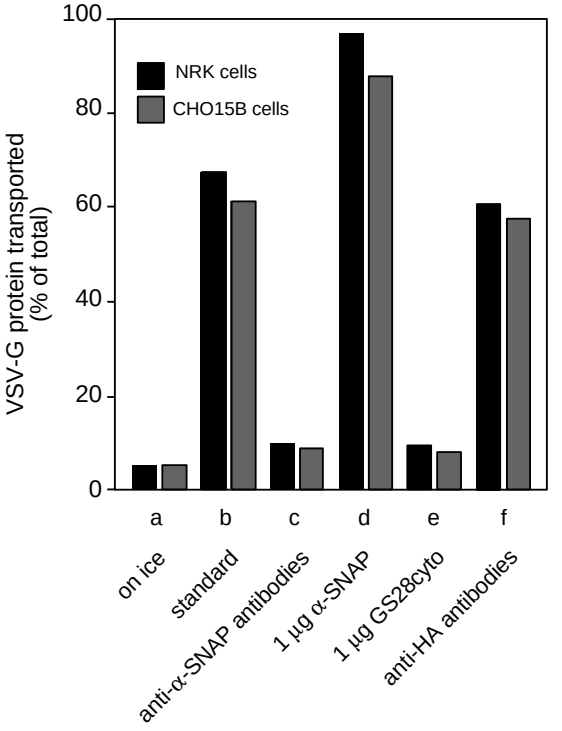


Fig. 3. Antibodies against α-SNAP inhibit while recombinant α-SNAP enhances transport from the ER to the *cis*-Golgi. VSV infected and permeabilized NRK cells (black bars) or CHO15B cells (grey bars) were incubated in a complete transport cocktail including ATP and rat liver cytosol for 90 minutes on ice (bars a) or at 32°C (bars b-f) under the following conditions: bars a, on ice; bars b, standard transport without additions; bars c, 2 μg anti α-SNAP antibodies; bars d, 1 μg recombinant α-SNAP; bars e, 1 μg recombinant GS28cyto (Subramaniam et al., 1996); bars f, 5 μg anti-haemagglutinin (HA) antibodies. Processing of the carbohydrate chains of the VSV-G protein to their endoglycosydase H (endo H) resistant (NRK cells) or endoglycosidase D sensitive (CHO15B cells) forms was measured.

γ-SNAP is not essential for ER-Golgi transport

Since γ-SNAP is structurally related (with 25% amino acid identity) to α-SNAP (Whiteheart et al., 1993), we also examined the functional importance of γ-SNAP. We prepared cytosol that was immunodepleted of γ-SNAP (Fig. 6A, lane 2), while cytosol immunodepleted of α-SNAP (lane 1), Rab1 (lane 3) or mSEC13 (lane 4) or mock-depleted (lane 5) contained normal levels of γ-SNAP. As shown in Fig. 6B, ER-Golgi transport was neither inhibited by antibodies against γ-SNAP (e) nor stimulated by recombinant γ-SNAP (d). Furthermore, cytosol immunodepleted of γ-SNAP supported normal transport (c). These results demonstrate that, in contrast to α-SNAP, γ-SNAP is not essential for ER-Golgi transport and can not substitute for α-SNAP in this transport event.

α-SNAP action precedes a step that is blocked by EGTA

To understand the mechanistic aspects of α-SNAP action in ER-Golgi transport, we have attempted to map the stage at which it acts. Previous studies using both mammalian as well as yeast cells have clearly established that EGTA, a Ca^{2+}-chelating agent, can block ER to *cis*-Golgi transport at a step that is after vesicle docking but before the actual event of membrane fusion between the docked vesicles and the *cis*-Golgi membrane (Aridor et al., 1995; Balch et al., 1994;

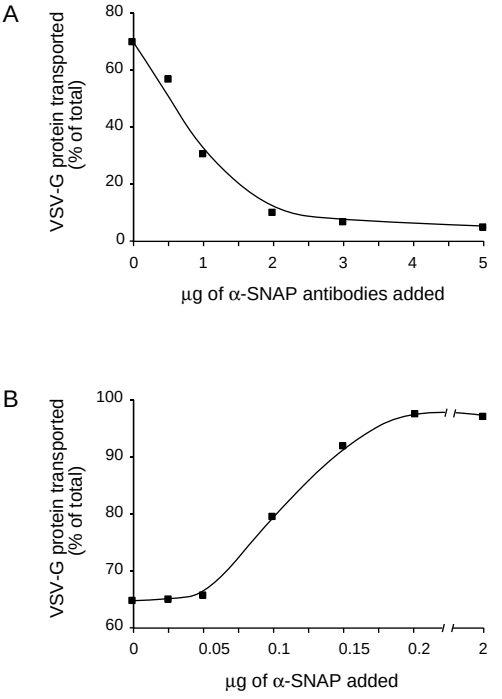


Fig. 4. Dose-dependent effects of α-SNAP antibodies (A) and recombinant α-SNAP (B) on ER-Golgi transport. Permeabilized NRK cells, ATP and rat liver cytosol were supplemented with increasing amounts of α-SNAP antibodies (A) or recombinant α-SNAP (B), preincubated for 60 minutes on ice and subsequently incubated for 90 minutes at 32°C. Processing of the VSV-G carbohydrate chains to the endo H resistant form was measured.

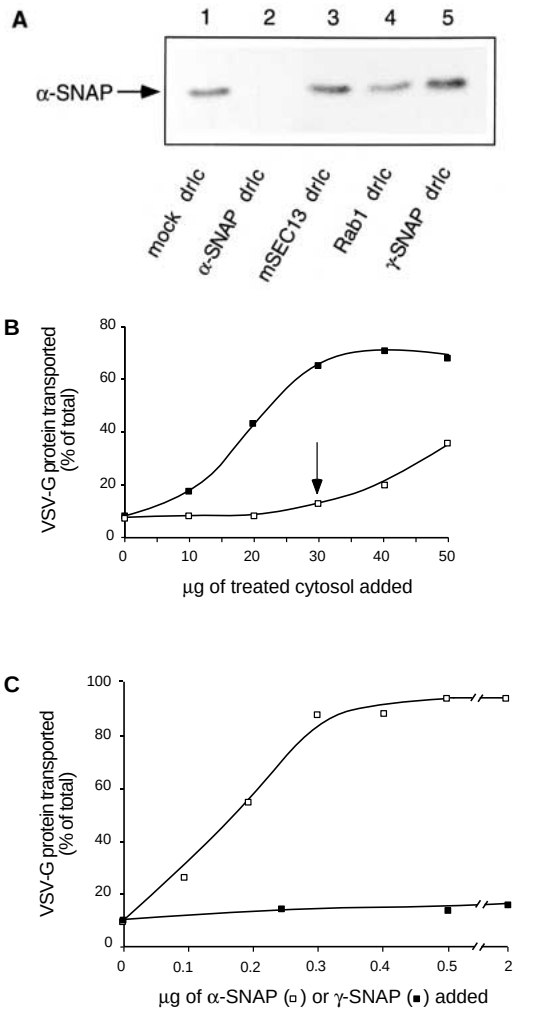


Fig. 5. α-SNAP is necessary for ER-Golgi transport. (A) 30 μg of cytosol that has been mock-depleted (lane 1), or immunodepleted with antibodies against α-SNAP (lane 2), mSEC13 (lane 3), Rab1 (lane 4), or γ-SNAP (lane 5) was analysed by immunoblot with antibodies against α-SNAP. (B) α-SNAP-depleted (open squares) or mock-depleted cytosol (filled squares) was tested for their ability to support ER to Golgi transport in vitro using permeabilized NRK cells, ATP and the respective depleted rat liver cytosol (drlc) were incubated for 90 minutes at 32°C and processing of VSV-G to its endo H resistant form was measured. 30 μg of α-SNAP drlc (arrow) were used for restoration studies outlined in C in which permeabilized NRK cells, ATP and 30 μg of α-SNAP drlc were supplemented with the indicated amounts of recombinant α-SNAP (open squares) or γ-SNAP (filled squares) and processing of VSV-G to its endo H resistant form was measured.

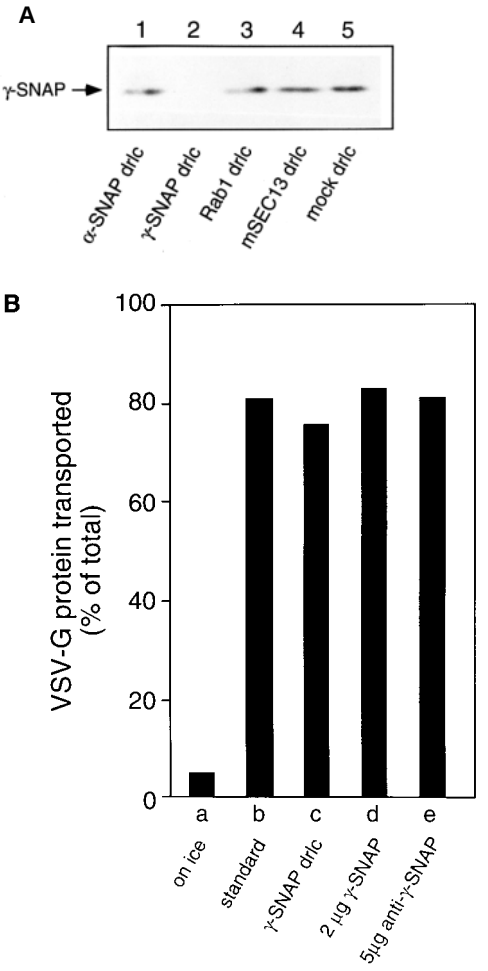
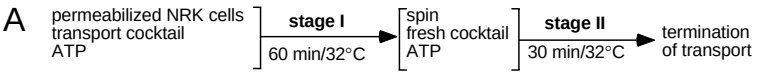


Fig. 6. γ-SNAP is not essential for ER-Golgi transport. (A) 30 μg of cytosol that has been immunodepleted with antibodies against α-SNAP (lane 1), γ-SNAP (lane 2), Rab1 (lane 3), mSEC13 (lane 4) or mock-depleted (lane 5) was analysed by immunoblot with antibodies against γ-SNAP. (B) In vitro ER-Golgi assay was performed on ice (a) or at 32°C with normal cytosol (b,d,e) or γ-SNAP depleted cytosol (c) supplemented with recombinant γ-SNAP (d) or γ-SNAP antibodies (e).

Lupashin et al., 1996; Pind et al., 1994; Rexach and Schekman, 1991; Subramaniam et al., 1996). The EGTA blockage is reversible and the actual fusion event can be restored after removal of EGTA and re-incubating in fresh cytosol. A two-stage transport assay was designed (Fig. 7A). Stage I was performed for 60 minutes at 32°C in defined cytosol and/or supplementing reagents, followed by a washing step and stage II incubation at 32°C for 30 minutes in the specified cytosol and/or supplementing additives were included. As shown (Fig. 7B), when EGTA was present in both stage I and stage II incubations, transport remained at close to background levels (I). Similarly, when α-SNAP depleted cytosol was used in both stage I and stage II incubations, no transport was detected (II). However, when α-SNAP depleted cytosol was used in stage I and normal cytosol was in stage II incubation, normal transport was achieved (III). When the assay was first performed in the presence of EGTA followed by stage II incubation with either normal (IV) or α-SNAP depleted cytosol (V), normal transport was observed, demonstrating that, like the control cytosol, α-SNAP depleted cytosol can restore transport from the EGTA blocked stage (EBS) to the actual fusion. These results suggest that α-SNAP is not required for events from the EBS to the actual fusion event but do not exclude the possibility that soluble α-SNAP is actually recruited during the stage I incubation in the presence of EGTA and functions during the stage II incubation, regardless of the presence or absence of soluble α-SNAP. To distinguish between these two possibilities, stage I incubation was perfomed in the presence



B

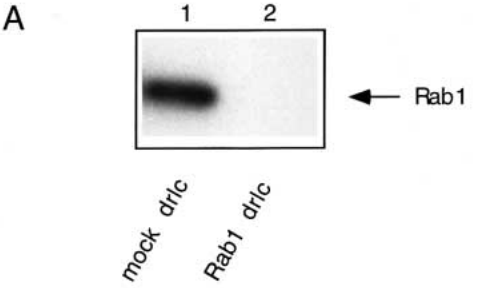
	stage I	stage II	VSV-G protein transported (%)
I	rlc + EGTA	rlc + EGTA	21
II	α-SNAP drlc	α-SNAP drlc	12
III	α-SNAP drlc	rlc	78
IV	rlc + EGTA	rlc	68
V	rlc + EGTA	α-SNAP drlc	70
VI	rlc + EGTA	rlc + α-SNAP antibodies	65
VII	α-SNAP drlc	rlc + EGTA	15
VIII	rlc + EGTA	rlc + recomb. α-SNAP	72

Fig. 7. α-SNAP is required before the EBS in ER-Golgi transport. Permeabilized NRK cells, ATP and either rat liver cytosol supplemented with 8 mM EGTA or α-SNAP depleted rat liver cytosol were incubated for 60 minutes at 32°C (stage I). Cells were then pelleted and resuspended in fresh buffer, ATP and the indicated cytosol (stage II). Processing of VSV-G to the endo H resistant form was measured.

of EGTA followed by stage II incubation with normal cytosol supplemented with 2.5 μg of α-SNAP antibodies (VI). As shown, normal transport was achieved, demonstrating that functional activity of α-SNAP could no longer be inhibited by antibodies during the EBS-to-fusion stage of transport. Additionally, when stage I transport was performed with α-SNAP depleted cytosol followed by stage II incubation with EGTA, no transport was observed (VII), demonstrating that α-SNAP depleted cytosol could not support transport to a step that is downstream of the EBS. Furthermore, when stage I incubation was performed in the presence of EGTA followed by stage II incubation with control cytosol supplemented with recombinant α-SNAP, no enhancing/stimulating effect was observed (VIII). These results demonstrate that α-SNAP is required for ER-Golgi transport before the EBS. Our results indicate that both the recruitment of α-SNAP and its functional involvement precede the EBS and that it might not play a role in the actual membrane fusion event.

Rab1 acts downstream of vesicle budding

Although members of Rab/Ypt1p/Sec4p family are generally believed to regulate vesicle docking and/or fusion (Novick and Brennwald, 1993; Nuoffer and Balch, 1994; Simons and Zerial, 1993), it was shown that Rab1 may actually participate in vesicle budding (Nuoffer et al., 1994; Peter et al., 1994a,b). To reexamine this issue independently, we have prepared cytosol immunodepleted of Rab1 (Fig. 8A) and performed a similar two stage assay for ER-Golgi transport (Fig. 8B and C). When cytosol immunodepleted of Rab1 (I) or mSEC13 (II) was used in both stages, only background levels of transport were detected, establishing that the respective depleted cytosols could not support normal transport. When mSEC13-depleted cytosol (which contains Rab1) was used for the first stage transport followed by the second stage in Rab1-depleted cytosol (which contains mSEC13), transport was not achieved (III) , suggesting that Rab1 could not act before mSEC13. In marked contrast, when Rab1-depleted and mSEC13-depleted cytosols

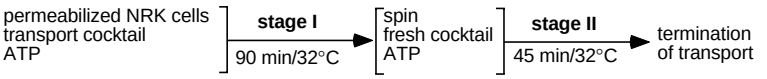


C

	stage I	stage II	VSV-G protein transported (%)
I	Rab1 drlc	Rab1 drlc	19
II	mSEC13 drlc	mSEC13 drlc	11
III	mSEC13 drlc	Rab1 drlc	12
IV	Rab1 drlc	mSEC13 drlc	64

Fig. 8. Rab1 acts downstream of mSEC13. (A) 30 μg of cytosol that had been mock-depleted (lane 1) or immunodepleted with a monoclonal antibody against Rab1 was analysed by immunoblot with polyclonal antibodies against Rab1. (B) Outline of the two-stage transport assay. (C) Stage I and II were performed with the indicated cytosol and the extent of transport was quantitated.

A



B

	stage I	stage II	VSV-G protein transported (%)
I	Rab1 drlc	α-SNAP drlc	15
II	α-SNAP drlc	Rab1 drlc	70
III	mSEC13 drlc	α-SNAP drlc	12
IV	α-SNAP drlc	mSEC13 drlc	74

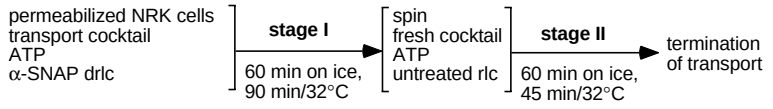
Fig. 9. α-SNAP is required after the Rab1 and mSEC13 requiring steps in ER to Golgi transport. Permeabilized NRK cells, ATP and the indicated cytosol were incubated for 90 minutes at 32°C (stage I). The membranes were pelleted and subsequently resuspended in fresh assay cocktail plus the indicated cytosol. The incubation was continued for an additional 45 minutes at 32°C (stage II).

were used for the first and second stage (IV), respectively, normal transport was achieved, suggesting that mSEC13 action precedes that of Rab1. Since mSEC13 is a component of COPII coat for vesicle budding (Barlowe et al., 1994; Salama et al., 1993; Shaywitz et al., 1995; Tang et al., 1997), our results suggest that Rab1 acts downstream of vesicle budding.

α-SNAP is required at a step after vesicle budding and the Rab1-requiring step

Although we elucidated that α-SNAP action precedes the EBS, the exact stage of its involvement between the budding stage to the EBS remained undefined. To gain additional understanding about its mechanistic aspects of action, we used the two-stage transport assay and mapped the requirement for α-SNAP to be downstream of the budding stage and the step that requires Rab1 (Fig. 9). We prepared cytosols that were immunodepleted of either mSEC13 (Tang et al., 1997) or Rab1 (Fig. 8A) and thus rendered defective in supporting ER-Golgi transport. When the transport assay was first performed using Rab1-depleted (α-SNAP-containing) cytosol followed by α-SNAP depleted (Rab1-containing) cytosol, no transport was observed (I). However, normal transport occurred when the assay was first performed with α-SNAP depleted (Rab1-containing) cytosol followed by Rab1 depleted (α-SNAP-containing) cytosol (II). These results suggest that α-SNAP acts at a step downstream of the Rab1-requiring step, a critical step in ER-Golgi transport prior to vesicle docking/fusion (Nuoffer and Balch, 1994; Pind et al., 1994). When the two-stage assay was first performed with mSEC13-depleted (α-SNAP-containing) cytosol followed by α-SNAP depleted (mSEC13-containing) cytosol, transport did not occur (III). Normal transport was achieved when the assay was performed with α-SNAP depleted (mSEC13-containing) cytosol followed by mSEC13 depleted (α-SNAP-containing) cytosol (IV), demonstrating that α-SNAP action is also downstream of the mSEC13-requiring step. These results suggest that α-SNAP action is required after the vesicle budding stage and the Rab1-requiring step. To gain independent evidence to support our conclusion, we performed experiments outlined in Fig. 10. Stage I incubation was performed with α-SNAP depleted cytosol followed by stage II incubation with normal cytosol, which led to normal transport (I). Inhibitory antibodies against mSEC13 or Rab 1 were included in either stage I or stage II incubation. When included in stage I incubation, antibodies against mSEC13 (III) or Rab1(II) inhibited the transport. In marked contrast, the antibodies exhibited no inhibitory effects when supplemented in the stage II incubation (IV-V), demonstrating that stage I incubation with α-SNAP depleted cytosol could drive the transport to a stage that is no longer inhibited by antibodies against mSEC13 or Rab1. Taken together, these results suggest that α-SNAP acts downstream of both vesicle budding and the Rab1-requiring step.

A



B

	stage I	stage II	VSV-G protein transported (%)
I	—	—	78
II	Rab1 antibody	—	18
III	mSEC13 antibodies	—	10
IV	—	Rab1 antibody	74
V	—	mSEC13 antibodies	81

Fig. 10. Antibodies against Rab1 and mSEC13 inhibit ER-Golgi transport before the α-SNAP-requiring step. Permeabilized NRK cells, ATP and α-SNAP depleted rat liver cytosol were incubated in the absence or presence of the indicated antibodies for 60 minutes on ice to allow proper diffusion of the antibodies into the permeabilized cells. Subsequently the incubation was continued for 90 minutes at 32°C. The membranes were then pelleted and resuspended in fresh cocktail including ATP, untreated rat liver cytosol and the indicated antibodies. After 60 minutes on ice the assays were incubated for 45 minutes at 32°C and transport measured as above.

DISCUSSION

An essential role for α-SNAP but not γ-SNAP in ER-Golgi transport

Our report presents the first detailed study that experimentally establishes a clear role for α-SNAP in vesicular transport from the ER to the *cis*-Golgi. Three independent sets of data support this point. The first line of evidence is that antibodies against α-SNAP exhibited quantitative inhibition of ER-Golgi transport. This inhibition is most likely due to the binding of antibodies to endogenous α-SNAP in the transport assay because this inhibition could be neutralized by exogenous recombinant α-SNAP (data not shown). Secondly, recombinant α-SNAP has an enhancing/stimulating effect on ER-Golgi transport, suggesting that α-SNAP is a limiting factor in the in vitro transport system. Thirdly, cytosol immunodepleted of endogenous α-SNAP is defective in transport and this defect could be rescued by the addition of recombinant α-SNAP. A previous study has observed a similar enhancing/stimulating effect of recombinant α-SNAP on ER-Golgi transport (Ikonen et al., 1995). However, that study neither investigated whether α-SNAP antibodies could inhibit transport nor did it establish if endogenous α-SNAP was necessary for ER-Golgi transport. In our system, the addition of 0.2 μg or more of recombinant α-SNAP could enhance the transport to maximal levels when normal cytosol was used for transport, while 0.3-0.4 μg of recombinant α-SNAP was required to restore α-SNAP depleted cytosol to maximal levels of transport. Hence it could be estimated that endogenous levels of α-SNAP may be around 0.1-0.2 μg in the amount of cytosol added per transport reaction. Interestingly, it was observed that excess α-SNAP (even >10 times over the amount required for maximal transport) had no adverse effect on transport. This indicates that free α-SNAP does not inhibit the function of α-SNAP-containing protein complexes and that its role may actually be to promote complex formation. Although γ-SNAP is structurally homologous to α-SNAP, recombinant γ-SNAP could not restore transport activity of α-SNAP-depleted cytosol. Furthermore, our results also clearly demonstrate that γ-SNAP is not essential for ER-Golgi transport.

Rab1 acts downstream of vesicle budding

Previous studies suggest that Rab1 may participate in vesicle budding from the ER because it acts at the same stage as COPI (Nuoffer et al., 1994; Peter et al., 1994a,b). Recent studies establish that COPI is not involved in budding from the ER but rather involved in an event downstream of vesicle budding mediated by COPII (Aridor et al., 1995; Scales et al., 1997). These previous studies could thus be re-interpreted to suggest that Rab1 acts downstream of vesicle budding from the ER. We have further investigated this issue using cytosol that was immunodepleted of Rab1 or mSEC13 in a two-stage transport assay, we have shown that mSEC13 must act before Rab1 for normal transport to be achieved. When mSEC13-depleted cytosol (which contains Rab1) was added before Rab1-depleted cytosol (which contains mSEC13), transport could not be detected. Our results thus clearly establish that Rab1 acts in an event after vesicle budding from the ER and are in agreement with other studies showing participation of Rab1 in a critical step prior to vesicle docking/fusion (Nuoffer and Balch, 1994; Pind et al., 1994; Simons and Zerial, 1993).

α-SNAP probably functions during the pre-docking or docking process

Mechanistic aspects of the involvement of α-SNAP in ER-Golgi transport were investigated by mapping the stage of its action. Using EGTA to block the ER to *cis*-Golgi transport at a stage that is after vesicle docking but before the actual fusion event (Aridor et al., 1995; Balch, 1994; Lupashin et al., 1996; Pind et al., 1994; Rexach and Schekman, 1991; Subramaniam et al., 1996), we have demonstrated that α-SNAP is required/recruited before the EBS. This was demonstrated by the observation that events after the EBS could be supported by α-SNAP depleted cytosol. Additional evidence that both the recruitment of α-SNAP and its action precede the EBS comes from the fact that transport from the EBS to the actual fusion is not inhibited by α-SNAP antibodies and that recombinant α-SNAP do not exhibit an enhancing/stimulating activity on the transport when supplemented during the EBS-fusion assay. The α-SNAP action has been further mapped to a step downstream of vesicle budding and the Rab1-requiring step as normal transport could be achieved when the assay was first performed with α-SNAP depleted (mSEC13- and Rab1-containing) cytosol followed by either mSEC13 or Rab1 depleted (α-SNAP-containing) cytosol and since α-SNAP depleted cytosol could drive the transport to a stage that is no longer inhibited by antibodies against mSEC13 or Rab1. From all these data, we conclude that α-SNAP acts after vesicle budding and the Rab1-requiring step but before the EBS. This mode of action for α-SNAP is in general agreement with that proposed by the SNARE hypothesis with some variations. The agreement lies in that recruitment and action of α-SNAP occur after vesicle budding, that Rab1 may regulate the α-SNAP-requiring step, and that α-SNAP is involved in the docking step (Rothman, 1994; Rothman and Warren, 1994; Rothman and Wieland, 1996; Söllner et al., 1993). Although we do not have a clear demonstration of the involvement of α-SNAP in the actual docking process, the most obvious event between the vesicle budding/Rab1-requiring step and the EBS is the pre-docking activation of SNAREs and the docking of vesicles with the *cis*-Golgi membrane. Therefore, it seems likely that α-SNAP recruitment and action are both at the pre-docking and/or docking stage during ER to *cis*-Golgi transport and could potentially be subject to regulation by Rab1. Our results also indicate that α-SNAP may not play a role during vesicle fusion with the *cis*-Golgi membrane, an observation at variation with the proposed role of α-SNAP in the fusion process as in the light of the SNARE hypothesis. Of course, our results do not completely exclude the possibility that α-SNAP may have a regulatory role for the fusion and that this regulatory role is exercised during the docking stage and before the EBS. More studies are needed to define the precise role of α-SNAP in ER-Golgi transport. In view of recent demonstration that SNAREs are the minimal machinery involved in membrane fusion (Weber et al., 1998) and that yeast Sec18p and Sec17p (Mayer et al., 1996) are involved in the pre-docking event during homotypic fusion of vacuoles, it seems most likely that α-SNAP is involved in the pre-docking event to activate the SNAREs.

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